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Low expression of microphthalmia-associated transcription factor, a potential molecular target for interferon-alpha susceptibility, is associated with metastasis in renal cell carcinoma

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We previously reported that microarray expression profiling identified several candidate genes in association with interferon-α (IFN-α) response in renal cell carcinoma (RCC) cell lines (Cancer Sci 2007; 98: 529). Among them, we focused on microphthalmia-associated transcription factor (*MITF*), because its expression profile correlated well with IFN-α-response status. In addition, we investigated the clinical significance of the expression level of *MITF* using surgical specimens. RNA was extracted from 14 RCC cell lines and 65 RCC samples and was used in this study. Transfection of *MITF* cDNA into IFN-α-resistant RCC cell lines resulted in elevation of *MITF* expression and acquisition of IFN-α-sensitivity by quantitative PCR and WST-8 assay, respectively. Statistical analysis revealed that low *MITF* mRNA expression in RCC samples was significantly correlated with the presence of metastasis and poor survival of the patient. However, the correlation between *MITF* expression and IFN-α response was not obvious in the clinical cases. *MITF* gene transfection elevated IFN-α-sensitivity in RCC cell lines, suggesting that this gene is a target molecule for modulation of the IFN-α response. Quantification of *MITF* mRNA expression might be clinically useful to predict metastasis and survival of patients with RCC. (*Cancer Sci* 2009; 100: 1714–1718)

The clinical course of renal cell carcinoma (RCC) is unpredictable, and the mean survival (approximately 8–12 months) of patients with advanced disease has not significantly increased during the last 20 years, despite the implementation of interleukin-2 (IL-2), interferon-α (IFN-α), and combination therapies.[1,2] Recently the molecular targeting drugs sorafenib, sunitinib, and temslorimus were approved for treatment of advanced RCC due to a higher response rate and higher stabilization of disease progression.[3,4] However, several clinical trials have shown a lower complete response rate and less synergistic effect with IFN-α. In this study, we aimed to investigate the effects of a new candidate molecular target, microphthalmia-associated transcription factor (*MITF*), which might be associated with IFN-α response using microarray expression analysis.[5] *MITF* is located at chromosome 3p14.2–14.1 and binds to the hypoxia-inducible factor 1α (HIF-1α) promoter. It up-regulates vascular endothelial growth factor (VEGF), similar to the status of von Hippel–Lindau (VHL) protein mutation.[6] *MITF* is also considered to be a transcription regulator because it binds to the $p16^{INK4a}$ (p16) promoter to activate p16 and inhibit phosphorylation of retinoblastoma protein (pRb).[7] Therefore, *MITF* not only plays a role as a potential trigger of clear cell RCC carcinogenesis and development, but also as a regulator of the cell cycle or proliferation of RCC, particularly through response to IFN-α. Thus, in this study we investigated whether or not susceptibility to IFN-α can be elevated by gene transfection of *MITF* in IFN-α-resistant RCC cell lines. In addition, we analyzed the relationships between *MTIF* expression levels and clinicopathological parameters, including patient prognosis, to reveal the clinical significance of *MITF* in RCC using surgically resected RCC specimens.

Materials and Methods

Renal cell carcinoma (RCC) cell lines and RNA extraction. Fourteen species of SKRC cell lines (SKRC-1, -6, -10, -12, -17, -24, -29, -33, -35, -59, SN12C (kind gift from Dr. S Naito, Fukuoka, Japan), THUR10TKB, THUR25TKB, and OS-RS-2) were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% L-glutamine in an incubator at 37°C and 5% CO_2 atmosphere. First, the IFN-α sensitivity of the cells was evaluated as described in the following section, and then the expression of *MITF* was analyzed.

At 80% confluence ($5–10 \times 10^6$ cells) and before addition of IFN-α, the RNA was extracted from each cell line. Briefly, cells were washed with phosphate-buffered saline (PBS) and lysed with 1 mL of TRIzol reagent (a phenol and guanidine isocyanate solution; Gibco BRL, Grand Island, NY, USA). Chloroform (200 μL) was added, and the mixture was centrifuged at 4°C and 12 000 *g* for 15 min. The liquid phase was precipitated with isopropanol. The RNA pellets were dissolved in Tris EDTA (TE) buffer. In addition, RNA was extracted from the selected cell lines at 3, 6, and 12 h after IFN-α treatment. The cell line RPTEC 5477–1 (Clonetics, San Diego, CA, USA) derived from epithelial cells of the human renal proximal tubule was used as a control for RNA expression.

Patients and RNA extraction. Between 1999 and 2007, the 65 RCCs and corresponding normal samples analyzed in this study were collected during surgical procedures. Ethical approval and prior patient consent were obtained in each case. The surgical specimens were snap-frozen in liquid nitrogen and stored at –80°C until use. The corresponding patient characteristics are summarized in Table 1. Tumor stages and pathological grades were assigned according to the general rules developed by the Japanese Urology Association, the Japanese Society of Pathology, and the Japan Radiological Society (3rd Edition). These rules follow the criteria of histological cell types and TNM staging of the International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC).[8,9] Pathological grades were assigned according to a system developed by the Japanese Urological Association

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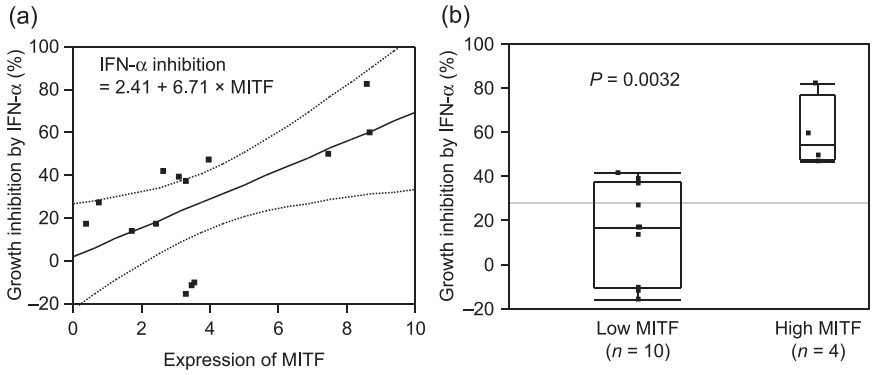

Fig. 1. Correlation between expression of microphthalmia-associated transcription factor (*MITF*) and interferon (IFN)-α susceptibility in 14 renal cell carcinoma (RCC) cell lines. (a) RCC cell lines expressing higher *MITF* mRNA demonstrated statistically higher growth inhibition by IFN-α. (b) IFN-α susceptibility was statistically different between high and low expressions of *MITF* mRNA in RCC cell lines ($P = 0.0032$).

Table 1. Characteristics of patients with RCC

Age: average/median (range)	62.9/62.0 (34–86)
Sex: male/female	48/17
Stage	
I/II/III/IV	29/4/14/18
T1/T2/T3/T4	33/7/22/3
Metastasis yes/no	17/48
Histology	
Clear/non-clear	53/12
Grade 1/2/3	5/36/24
Interferon-α	
No/Therapeutic/Adjuvant	51/10/4
Response CR/PR/SD/PD	0/1/3/6
MITF expression	
Median (range)	0.366 (0.017–3.39)

CR, Complete response; *MITF*, microphthalmia-associated transcription factor; PD, Progression disease; PR, Partial response; RCC, renal cell carcinoma; SD, Stable disease.

based on the nuclear size and degree of atypia of tumor cells. The tumors were classified as low grade (grade 1) when they had smaller nuclei than normal epithelial cells (normal), moderate (grade 2) when they showed nuclei equal in size to normal, or high (grade 3) when they showed larger and more polymorphic nuclei than normal. Total RNA was extracted from each sample by the procedure described above.

Quantitative RT-PCR. Quantitative RT-PCR was performed using the TaqMan Gene Expression assay and an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: initial incubation at 50°C for 2 min and denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. RNA expression levels were compared using an RNA pool from 40 samples of a normal kidney parenchyma. The 18-s RNA was used as an internal control. Expression of *MITF* mRNA was measured by quantitative RT-PCR using primers and TaqMan probe sequences that were designed using Assays-on-Demand Gene Expression probes (Applied Biosystems) according to the assay ID corresponding to the GenBank Public ID for *MITF*.

Correlation between expression of *MITF* mRNA and IFN-α response in RCC cell lines. All RCC cell lines were treated with 3000 IU/mL of IFN-α at the final concentration as reported previously.[5] Then, the growth inhibition was assessed on the 5th day after treatment using a WST-8 assay (Dojindo, Tokyo, Japan) according to the manufacturer's directions. The WST-8 assay was repeated at least five times on each cell line to obtain reproducible results. The inhibition rate was calculated by comparing the average inhibition with that of the control (without IFN-α treatment) in each cell line. To compare the *MITF* expression level with IFN-α susceptibility, cells were divided into two groups with the cut-off level at the average level of *MITF* expression in this series. Statistical analysis was performed to obtain the correlation coefficient, and ANOVA was used to show the difference between IFN-α responses of *MITF*-low and -high cell lines.

Transfection of *MITF* cDNA into RCC cell lines. We focused on alterations of IFN-α susceptibility after over-expression of *MITF* in the IFN-α-resistant RCC cell line SKRC-33. For the transfection study, *MITF* cDNA (MHS1010-9206145; Open Biosystems, Huntsville, AL, USA) was cloned into pcDNA 3.1(+) using EcoRI on both sides, then *MITF* cDNA was transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Tokyo, Japan) After transfection of the gene, increased expression of *MITF* was confirmed by quantitative RT-PCR as mentioned above.

Comparison of *MITF* expression and IFN-α response before and after transfection. The IFN-α response was evaluated by growth inhibition after IFN-α treatment using WST-8 assay as previously described. Cells were treated with two-stage dilutions of IFN-α from 300 to 1000 IU/mL at the final concentration, and then the growth inhibition was assessed at 3 and 5 days after treatment. The WST-8 assay was repeated at least five times on each cell line. To evaluate the effect of *MITF* transfection, the wild type, *MITF* transfectant, and a mock transfectant of SKRC-17 and -33 were analyzed.

Correlation between expression of *MITF* mRNA and clinicopathological parameters in patients with RCC. The *MITF* mRNA expression level was analyzed in clinical samples in association with clinicopathological parameters, i.e. stage, grade, presence of metastasis, and IFN-α response, during the follow-up period (range, 1.5 to 93.6 months; median, 36.1 months) according to the patient records. Survival analyses were also carried out according to *MITF* mRNA expression levels. In the statistical analysis, ANOVA and Student's *t*-test were used to evaluate the differences between groups, and the Kaplan–Meier method and log-rank test were used for survival analysis. In addition, Cox's proportional hazard analysis was used to reveal the hazard ratio of several parameters. These statistical procedures were supported by JMP 5.1.

Results

Response to IFN-α according to *MITF* expression in RCC cell lines. Growth inhibition rates in several RCC cell lines due to IFN-α according to expression of *MITF* mRNA are shown in Table 2 and Figure 1a. The interferon-α response seemed to be associated with the *MITF* expression level in both primary and established RCC culture cells. When the two groups were divided by the average expression level of *MITF*, growth inhibition by IFN-α in the high *MITF* expression group was significantly stronger than that in low expression group ($P = 0.0032$, Fig. 1b).

Transfection of *MITF* cDNA into SKRC-17 and -33. After transfection, both SKRC-17 and -33, which are IFN-α-sensitive and -resistant, respectively, successfully expressed *MITF* mRNA at higher levels

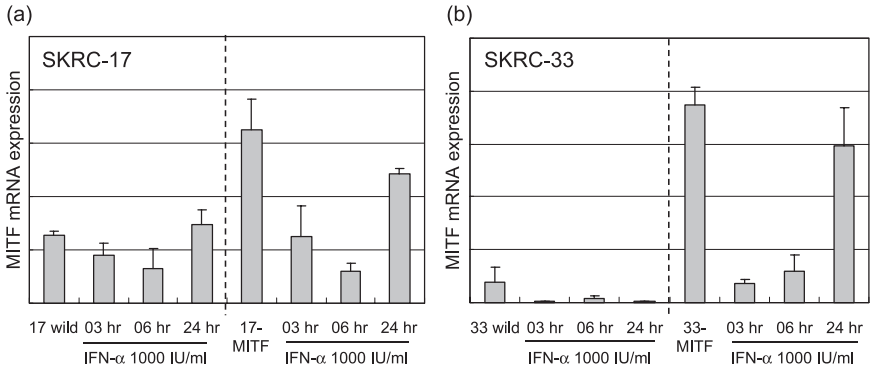

Fig. 2. Expression of microphthalmia-associated transcription factor (*MITF*) in the renal cell carcinoma (RCC) line after transfection of *MITF* cDNA. (a) After transfection of *MITF* cDNA, mRNA of *MITF* was overexpressed in SKRC-17, an interferon (IFN)-α-sensitive cell line using quantitative RT-PCR. (b) After transfection, the SKRC-33, an IFN-α-resistant cell line, expressed a high level of *MITF* mRNA as compared with the wild-type SKRC-33. After treatment with 1000 IU/mL IFN-α, expression of *MITF* decreased at 3 h, then increased again in both SKRC lines.

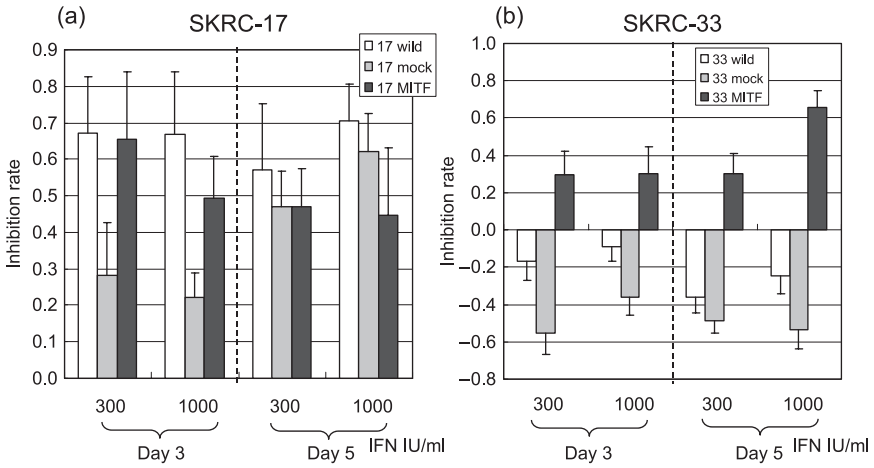

Fig. 3. The interferon (IFN)-α response in renal cell carcinoma (RCC) cell lines according to the expression status of microphthalmia-associated transcription factor (*MITF*). (a) In SKRC-17, which is an IFN-α-sensitive line, no remarkable difference was observed in IFN-α response among the wild type, mock transfectant, and *MITF* transfectant. (b) In an *MITF* transfectant of SKRC-33, INF-α inhibited cell growth independent of the IFN-α concentration as compared with the wild type or mock transfectant. Transfection of pcDNA 3.1(+) vector, i.e. mock transfection, did not inhibit cellular growth by itself but seemed to decrease IFN-α-based growth inhibition.

Table 2. Correlation between *MITF* expression and growth inhibition by IFN-α treatment

RCC cell lines	*MITF* expression	Growth inhibition by IFN-α
SKRC-1	2.66	41.6
SKRC-6	3.99	46.7
SKRC-10	3.32	36.8
SKRC-12	1.74	13.6
SKRC-17	8.69	59.8
SKRC-24	2.45	16.8
SKRC-29	3.56	−10.1
SKRC-33	0.399	17.1
SKRC-35	0.758	26.8
SKRC-59	7.46	49.5
SN12C	3.08	38.9
THUR10TKB	8.58	82.7
THUR25TKB	3.31	−15.7
OS-RS-2	3.47	−11.6

IFN-α, interferon-alpha; *MITF*, microphthalmia-associated transcription factor; RCC, renal cell carcinoma.

than the wild type (Fig. 2). When IFN-α was added at 1000 IU/mL, expression of *MITF* was decreased at 3 h, but subsequently recovered in both SKRC-17 and -33.

Alteration of IFN-α response after *MITF* gene transfection into RCC cell lines. In SKRC-17, no significant difference in IFN-α response was shown among the wild type, *MITF* transfectant, and mock-transfectant by WST-8 assay. On the other hand, the *MITF* transfectant of SKRC-33 revealed IFN-α susceptibility to IFN-α treatment at both 300 and 1000 IU/mL as compared with the wild type and mock transfectant (Fig. 3). As shown in Figure 3, transfection did not inhibit cellular growth by itself, because the mock transfectant seemed to demonstrate low IFN-α effect rather than wild type.

Expression of *MITF* and its significance in association with clinicopathological parameters in patients with clear cell RCC. Clinical samples of clear cell RCC seemed to express *MITF* mRNA at relatively lower levels than the normal kidney. Classification of patients into high and low *MITF* groups according to the median expression level of *MITF* mRNA revealed that expression of *MITF* was significantly lower in patients with distant and/or lymph node metastasis among several clinical parameters than in those without metastasis ($P = 0.0001$, Fig. 4a). In addition, patients with a high level of *MITF* expression in their RCC had a better prognosis than those with a low level using the median MITF expression (0.366) as a cut-off ($P = 0.0065$, Fig. 4b). However, no significant difference was observed in 10 patients between the *MITF* expression level and IFN-α response, which were none, one, three, and six out of 10 patients in complete response, progression disease, stable disease, and partial response, respectively ($P = 0.611$). No statistical difference was detected in survival curves in association with the *MITF* expression level in stage IV patients who received IFN-α treatment ($P = 0.826$, Fig. 5). Cox's proportional hazard analysis showed that RCC patients with low expression of *MITF* had 1.66-times higher risk of death than patients with high expression. However, stage and grade showed higher hazard ratio (HR) than *MITF* expression (HR = 6.64 between grade 2 and 3, and 2.66 between stage III and IV, respectively).

Discussion

Interferon-α is still a therapeutic option for RCC patients, but the response rate is only 15–20%,[1,2] and it has been reported

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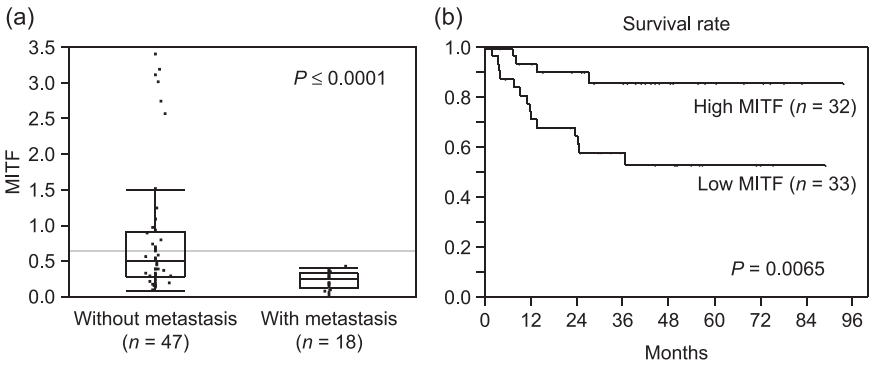

Fig. 4. Microphthalmia-associated transcription factor (*MITF*) mRNA expression and metastatic status in renal cell carcinoma (RCC) samples. (a) Clinical analysis revealed that primary renal tumors from patients with metastasis had statistically lower *MITF* mRNA expression than those without metastasis ($P < 0.0001$). (b) Patients with high *MITF* expression RCC had a better prognosis than those with low *MITF* ($P = 0.0065$). Median *MITF* expression level (0.366) among patients was used as a cut-off value between the two groups.

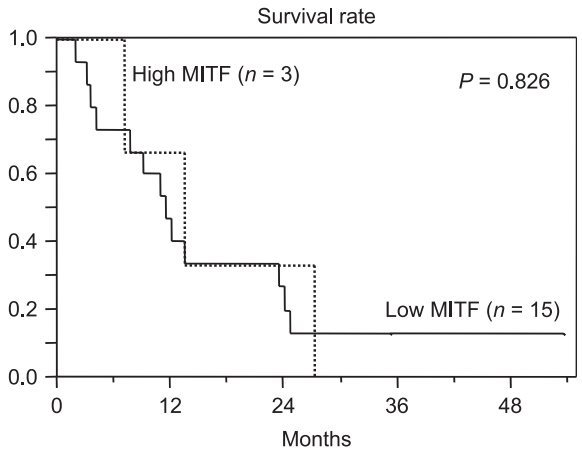

Fig. 5. Survival of patients who received therapeutic interferon (IFN)-α according to microphthalmia-associated transcription factor (*MITF*) expression pattern. Among patients who received therapeutic IFN-α, high or low expression of *MITF* mRNA had no prognostic value ($P = 0.826$).

that treatment with IFN-α has several adverse side effects.[10] The anti-tumor effects of IFN-α consist of the direct inhibition of tumor proliferation and the biological response modifiers (BRM). Because the molecular mechanisms of BRM are complex, we focused on investigation of the direct inhibition of cancer cells by IFN-α in this study.

The molecular mechanism of IFN-α resistance remains to be fully elucidated not only in RCC but also in other cancers, e.g. malignant melanoma and lymphoma, that are resistant to IFN-α. Defective Jak-STAT pathways[11] and loss of ISGF3 components,[12] which are key molecules in the IFN-α signal transduction pathway, have been identified *in vitro* in human melanoma cell lines as molecular mechanisms of IFN-α resistance. It was also reported that a lack of STAT1 expression was associated with IFN-α resistance in a T-cell lymphoma cell line[13] and in RCC.[14] Although STAT1 is required for IFN-α-induced growth inhibition, gene alterations of the Jak-STAT pathway in RCC have not been reported frequently.

Recently, Shang *et al*. reported that defective Jak-STAT activation is associated with IFN-α resistance, and found that restoring Jak-STAT increases the susceptibility of RCC cells to IFN-α.[15] In our previous study, we did not find any RCC cell lines that demonstrated a lack of molecules in the Jak-STAT signaling pathway, and only one case has been reported in the literature.[14] However, whether or not this pathway is impaired should be evaluated first in association with IFN-α resistance, even in restricted RCCs. According to Figure 2, MITF is apparently not involved in an IFN-α signaling pathway such as Jak-STAT because expression of *MITF* seems to decrease transiently in the early phase after IFN-α treatment. Although IFN-α may directly inhibit *MITF* expression in RCC cell lines, IFN-α susceptibility seems to correlate with the restoration of *MITF* expression at 24 h after IFN-α treatment. Thus MITF might not be included into a lower stream of Jak-STAT signaling, but might participate and emphasize the growth inhibition of IFN-α signaling.

Korkola *et al*.[16] also identified four key genes that are associated with IFN-α resistance by microarray expression profiling using 23 samples from patients with metastatic RCC and knowledge of the IFN-α clinical response status. Interestingly, they found that two of the four genes were involved in the chromosome 4q arm, which was previously implicated as the site of several putative tumor suppressor genes in many cancers, including in the kidney. Microphthalmia-associated transcription factor is not involved in this chromosome arm but in chromosome 3p (14.2–14.1), which is closed to the *VHL* locus. Probably several hotspots are involved in IFN-α resistance or susceptibility in RCC.

It is known that *MITF* is associated with a childhood (or young adulthood) translocation subgroup of RCCs, which are characterized by the occurrence of recurrent chromosomal translocations, resulting in disruption and fusion of either the transcription factor E 3 (*TFE3*) or transcription factor E B (*TFEB)* genes, both members of the *MITF* family of basic helix-loop-helix/leucine-zipper transcription factor genes.[17] It is apparent that the *MITF* discussed in the present study is not associated with this type of tumor, but it is associated with functional aspects of clear cell RCC, such as regulation of the cell cycle and proliferation. This might indicate that *MITF* plays opposite roles in proliferation through activation of angiogenesis and senescence by cell cycle regulation. Although high expression of *MITF* was observed in aggressive melanoma,[18] our clinical analysis suggested that *MITF* expression may be associated with less aggressive RCC behavior, probably because cell cycle regulation is a dominant function in RCC. Because it is also true that expression of VEGF does not always enhance metastasis of RCC in animal models, up-regulation of VEGF may not be an essential role of *MITF*, at least at the advanced stage of RCC. Low susceptibility of metastatic lesions to IFN-α may be explained by the finding that no RCC with high *MITF* expression was seen in patients with metastasis. From a therapeutic point of view, enhancement of *MITF* expression of the tumor might elevate the IFN-α response in advanced RCC.

Because *MITF* is a transcriptional regulator for the p16 promoter[7] and loss of p16 expression is a frequent event in RCC (date not shown), IFN-α susceptibility can be increased by gene transfection or a functional peptide transfer of p16.[19] This kind of treatment strategy should be practical for patients with advanced RCC.

In conclusion, *MITF* is considered to be a candidate gene that may up-regulate IFN-α susceptibility in RCC cell lines. Based on the present clinical investigations, we speculate that a possible reason for the low response rate of IFN-α in RCC patients

might be explained by the low expression of *MITF* in those patients with metastatic disease. At the same time, the key molecules or molecular targeting agents involved in the mechanism of enhancement of IFN-α response should be clarified by solving the molecular signaling pathway in RCC.

Acknowledgments

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